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Combinatorial effects of *Flk1* and *Tal1* on vascular and hematopoietic development in the mouse

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Mouse embryos mutant for the VEGF receptor, VEGFR2, *Flk-1*, or *Kdr*, fail to form both endothelial and hematopoietic cells, suggesting a possible role in a common progenitor to both lineages. The transcription factor *Tal1* (*Scl*), is not expressed in *Flk1*^{-/-} embryos, consistent with a downstream role in the *Flk1* pathway. We tested whether expression of *Tal1* under the *Flk1* promoter was sufficient to rescue the loss of endothelial and hematopoietic cells in *Flk1* mutants. Only partial rescue of hematopoiesis and endothelial development was observed in vivo. However, *Flk1*^{-/-}*Tal1* embryonic stem (ES) cells were capable of blast colony formation in vitro at levels equivalent to *Flk1*^{+/-} heterozygotes. Ectopic expression of *Tal1* under the *Flk1* promoter in *Flk1*^{+/-} mouse embryos or ES cells caused no obvious pathology but increased the number of blast colony forming cells (BL-CFCs) and enhanced their hematopoietic potential. These single-cell-derived BL-CFCs also produced smooth muscle cells in vitro. Increased *Tal1* expression inhibited smooth muscle differentiation in this assay, whereas loss of *Tal1* promoted smooth muscle formation. We propose a model in which the combinatorial effects of *Flk1* and *Tal1* act to regulate cell fate choice in early development into hematopoietic, endothelial, and smooth muscle lineages.

[Keywords: Hemangioblast; VEGF signalling; SCL/TAL1; endothelial development; smooth muscle; hematopoiesis]

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The cardiovascular system is the first organ system to appear in the developing mouse embryo and is critical for midgestational viability. During development of the vascular system, there is a close association between endothelial precursors and primitive hematopoietic cells in the blood islands of the yolk sac. A similar close association is also seen in later intraembryonic regions where definitive hematopoietic precursors arise. The close spatial and temporal relationships between endothelial and hematopoietic cells led to the hypothesis that they originate from a common progenitor, the hemangioblast (Sabin 1920). Recent molecular and genetic evidence has lent support to this concept. Early hematopoietic cells and angioblasts express a number of genes in common, including *Flt1*, *Kdr* (commonly known as

Flk1), *Tal1* (*Scl1*), *Tie1*, and *Tie2* (Kallianpur et al. 1994; Dumont et al. 1995; Fong et al. 1996; Elefanty et al. 1999; Drake and Fleming 2000). Of all the genes expressed in this manner, only mutation of *Flk1* leads to complete absence of hematopoietic and endothelial cells in vivo (Shalaby et al. 1995). Chimeric analysis with *Flk1* null embryonic stem (ES) cells showed that *Flk1* is required cell-autonomously for migration of progenitors of hematopoietic and endothelial cells to the yolk sac and is also required for generation of definitive hematopoietic precursors (Shalaby et al. 1997). Because *Flk1* encodes a tyrosine kinase cell surface receptor for the vascular endothelial growth factor family of ligands (Millauer et al. 1993), this suggests that the VEGF signaling pathway, acting through the *Flk1* receptor, is critical for the early establishment of the endothelial and hematopoietic lineages and perhaps for their common progenitor. In vitro evidence to support this came from studying the differentiation of ES cells into embryoid bodies. Single *Flk1*⁺ cells from embryoid bodies can give rise to blast colonies (BL-CFCs) which produce both hematopoietic and endothelial cells in vitro (Kennedy et al. 1997; Choi et al.

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1998; Faloon et al. 2000), thus defining a common progenitor or hemangioblast.

The pathways downstream of Flk1 signaling in the hemangioblast are not yet clear, but the TAL1/SCL bHLH transcription factor has been suggested to be involved, on the basis of its expression pattern and some functional studies. *Tal1* expression is detected in the presumptive yolk sac region in the mid/late streak stage of mouse embryos (Drake et al. 1997; Elefanty et al. 1999), coincident with *Flk1*, and continues to be expressed in hematopoietic cells in later stages (Green et al. 1992; Elefanty et al. 1998). It is also expressed in angioblasts and endothelial cells at lower levels (Kallianpur et al. 1994; Elefanty et al. 1999). *Flk1*^{-/-} ES cell-derived mesodermal cells are unable to differentiate into hematopoietic precursors on OP9 cells and do not express *Tal1*, in contrast to wild-type ES cell-derived mesodermal cells (Hidaka et al. 1999). Gene targeting of *Tal1* shows that TAL1 is essential for the development of all hematopoietic cells (Robb et al. 1995, 1996; Shivdasani et al. 1995; Porcher et al. 1996). Although TAL1 is dispensable for initial endothelial development, it is required for later vascular remodeling (Visvader et al. 1998; Elefanty et al. 1999).

Despite this genetic evidence that *Tal1* is not required for early endothelial development, other experiments have suggested a closer relationship between *Tal1* function and hemangioblast development. In situ hybridization in zebrafish, as in mouse, suggests that *Tal1* expression is very similar to that of *Flk1* and may mark the hemangioblast (Gering et al. 1998). Overexpression of *Tal1* in the fish caused an increased number of both blood cells and blood vessels at the expense of somite and nephrotic tissues (Gering et al. 1998), and partially rescued the defects of both hematopoiesis and vasculogenesis in the *cloche* mutant (Liao et al. 1998). In addition, *Tal1* expression initiates shortly after *Flk1* in mouse embryoid bodies (Faloon et al. 2000), and cells doubly sorted for Flk1 and TAL1 expression are enriched for hemangioblasts, as assessed by the blast colony assay (Chung et al. 2002).

To further examine the relationship between *Flk1*, *Tal1*, and hemangioblast development, we investigated *Tal1* expression in *Flk1*^{-/-} embryos and found that it is absent, consistent with a downstream role in hemangioblast development. We then tested whether expression of *Tal1* under the *Flk1* promoter was sufficient to rescue the loss of endothelial and hematopoietic cells in *Flk1* mutants. Only partial rescue of hematopoiesis and endothelial development was observed in vivo. However, *Flk1*^{-/-}*Tal1*⁺ ES cells were capable of blast colony formation in vitro at levels equivalent to those of *Flk1*^{-/-} heterozygotes. In addition, ectopic expression of *Tal1* under the *Flk1* promoter in *Flk1*^{-/-} cells increased the number of BL-CFCs and enhanced their hematopoietic potential in vitro and in mouse embryos. We also demonstrated that single-cell-derived BL-CFCs can produce smooth muscle cells (SMCs), suggesting that there may be a common progenitor for blood, blood vessel, and SMCs. Increased *Tal1* expression inhibited smooth muscle differentiation

in this assay, whereas loss of *Tal1* promoted smooth muscle formation. We propose a model in which the combinatorial effects of *Flk1* and *Tal1* act to regulate cell fate choice in early development into hematopoietic, endothelial, and smooth muscle cells.

Results

Tal1 expression is absent in *Flk1* mutant embryos

Flk1 mutant embryos were dissected at embryonic day 8.5 (E8.5) and subjected to whole mount in situ hybridization with a probe to *Tal1*. No expression was observed (Fig. 1A), although the mutant embryos do express other markers of early endothelial and hematopoietic development, such as *Flt1* and CD34 (Shalaby et al. 1995). This suggested that *Tal1* expression might be dependent on *Flk1* signaling, and that its absence could be critical in causing the loss of endothelial and hematopoietic development in *Flk1* mutants. This led us to test the effect of forced expression of *Tal1* in *Flk1* mutant embryos.

Targeting *Tal1* into the *Flk1* locus

To generate ES cell lines expressing ectopic TAL1, a targeting vector was designed to knock the full-length *Tal1* cDNA into the *Flk1* locus (Fig. 1E). The vector was successfully recombined into the wild-type *Flk1* locus in both wild-type R1 ES cells and an ES cell line heterozygous for a loss-of-function *lacZ* knock-in allele of *Flk1* (Shalaby et al. 1995). In the latter case, the floxed *neo* gene in the *lacZ* insertion allele was removed by transient Cre excision prior to retargeting the wild-type allele with the *Tal1* knock-in vector. Two independent *Flk1*^{+/Tal1} and three independent *Flk1*^{-Tal1} lines were isolated and characterized (Fig. 1D; data not shown). *Flk1*^{+/Tal1} lines are heterozygous for a functional Flk1 receptor and should coexpress TAL1 in all Flk1-expressing cells. *Flk1*^{-Tal1} lines are functionally null for *Flk1*. However, they will express TAL1 in all the domains where Flk1 expression can be activated, because we have previously shown that expression of the *Flk1* locus does not depend on the presence of functional Flk1 protein (Shalaby et al. 1995).

Cell lines of both types were aggregated with wild-type tetraploid ICR embryos (Nagy et al. 1993) and allowed to develop to E8.5. The phenotype of the entirely ES cell-derived embryos was compared with that of control embryos derived by aggregation of *Flk1*^{-/-} ES cells (Fig. 1B,C; data not shown). Two lines of *Flk1*^{-Tal1} cells generated apparently normal embryos at E8.5 (data not shown). Three lines of *Flk1*^{+/Tal1} cells, on the other hand, generated embryos after tetraploid aggregation in which development was blocked at the same stage as embryos generated from the original homozygous *Flk1* *lacZ* knock-in lines. Comparison of *lacZ* staining patterns between *Flk1*^{-Tal1} and *Flk1*^{-/-} embryos revealed stronger staining in the *Tal1* knock-in lines in both the embryo and the yolk sac (Fig. 1B,C). Occasionally, small clumps

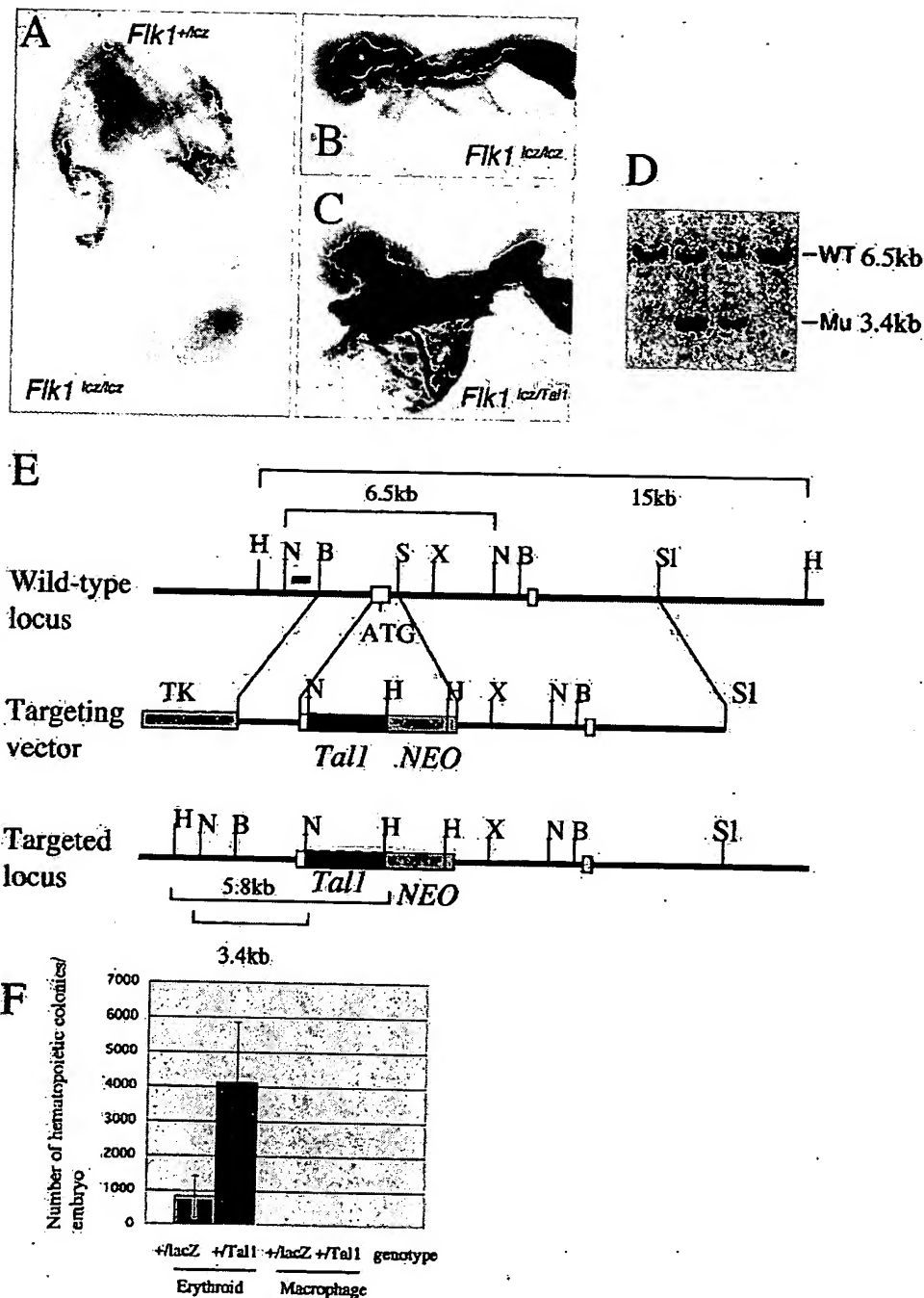


Figure 1. The construction of *Flk1-Tal1* knock-in vector, establishment of the ES cell lines, and expression of *Tal1* mRNA in *Flk1*^{lacZ/lacZ} embryos. (A) *Tal1* mRNA expression is missing in *Flk1*⁻ null embryos. *Tal1* mRNA is detected in the yolk sac, dorsal aorta, and allantois of 8.5-dpc wild-type embryo (top), but not in *Flk1*^{lacZ/lacZ} embryo (bottom). (B) X-gal staining of *Flk1*^{lacZ/lacZ} embryos. (C) X-gal staining of *Flk1*^{lacZ/Tal1} embryo. Note the marked increase of lacZ expression in the embryo as well as yolk sac. (D) Southern blot analysis of the established ES clones. ES DNAs were digested with NcoI. Wild-type band is 6.5 kb, mutant band is 3.4 kb. (E) The *Flk1-Tal1* knock-in vector. *Flk1* wild-type locus (top), targeting construct (middle), and targeted homologous recombination at the *Flk1* locus. The probe used in Southern blotting is shown as a black bar. ATG marks the start of translation of *Flk1*. (F) Hematopoietic colony assays on the yolk sac regions of E8.5 *Flk1*^{+/lacZ} (n = 8) and *Flk1*^{+/Tal1} (n = 6) embryos.

of hemoglobin-expressing cells and *Tie1*⁺ cells were seen in the yolk sac of the *Tal1* knock-in embryos (data not shown). However, in sections, most of the *lacZ*-positive cells in the embryo and yolk sac were neither endothelial nor hematopoietic (data not shown). This suggests that expression of *Tal1* in the *Flk1* locus is not sufficient to fully rescue hematopoietic or endothelial development in vivo.

Hemangioblast potential of *Tal1* knock-in line

The failure to rescue yolk sac hematopoiesis by expression of *Tal1* under the *Flk1* promoter could involve an intrinsic deficiency in hematopoiesis or failure of *Tal1* to rescue the defective migration of cells to the yolk sac that occurs in *Flk1* mutants. To distinguish these possibilities, we used the blast colony assay to assess the hemangioblast-like capacity of ES cells differentiating in vitro, where no migration to the correct microenvironment is required. Cells from day 2.75 embryoid bodies (EBs) were dissociated and replated in methylcellulose culture under blast colony conditions (Choi et al. 1998), and colonies were counted after 4 d of culture. All three *Flk1*^{+/Tal1} lines showed an increase in number of blast colonies compared to *Flk1*^{-/-} lines, to levels equivalent to *Flk1* heterozygous lines (Fig. 2A). Thus, expression of one allele of *Tal1* in place of one allele of *Flk1* is sufficient to rescue the hemangioblast defect in this in vitro assay but not in vivo.

Interestingly, however, under the same conditions, the heterozygous *Flk1*^{+/Tal1} lines showed a major increase in number of blast colonies compared to all other lines, including wild-type cells (Fig. 2A). To assess whether this effect was dependent on *Flk1*, the day 2.75 EBs from one *Flk1*^{+/Tal1} ES line and one *Flk1*^{-/-} line were sorted for *Flk1* expression by FACS (Fig. 2B), and *Flk1*-positive and -negative cells were grown under blast colony conditions. In both cases, very few blast colonies grew from the *Flk1*-negative populations, but in the *Flk1*-positive populations, the *TAL1* knock-in cells generated threefold more blast colonies than the *Flk1*^{-/-} cells (Fig. 2C). To determine whether the increase in blast colony potential was matched by an increase in hematopoietic potential, we performed hematopoietic colony assays on two independent *Tal1* knock-in lines. Both lines gave two- to three-fold higher numbers of erythroid, myeloid, and mixed colonies compared with control heterozygous lines (Fig. 2D). Hematopoietic colony assays on the yolk sac of *Flk1*^{+/Tal1} embryos derived from tetraploid aggregates also revealed increased hematopoietic potential. Cells from these embryos have a five-times higher number of primitive erythrocyte progenitors compared to control *Flk1*^{lacZ} heterozygous embryos (Fig. 1F).

Together these results suggest that, in the absence of *Flk1* signaling, expression of *TAL1* in *Flk1*⁺ precursors is not sufficient for proper hemangioblast development in vivo. However, increasing the percentage of cells that coexpress *Flk1* and *TAL1* by driving *Tal1* expression under the *Flk1* promoter can increase the proportion of hemangioblast-like cells in the *Flk1*-positive population in

vitro. Both observations suggest that coexpression of *Flk1* and *TAL1* is needed for proper hemangioblast development.

Differentiation potential of hemangioblast progenitors

Expression of *Tal1* under the *Flk1* promoter in *Flk1*^{+/Tal1} lines clearly increased the ability of the lines to form progenitors capable of generating hematopoietic cells. To determine whether ectopic expression of *TAL1* also affected the differentiation of other cell types from the *Flk1*-expressing hemangioblast, we established two culture conditions under which *Flk1*-expressing cells could develop into adherent endothelial and SMC progenitors, as previously reported by Yamashita et al. (2000). Populations of *Flk1*-positive and *Flk1*-negative cells were sorted from wild-type day 2.75 EBs with anti-*Flk1* antibodies. The purity of the *Flk1*-positive populations was over 99% (Fig. 2B). The same number of *Flk1*-positive and -negative cells were plated onto type IV collagen-coated 24-well dishes and cultured for 3 d. The attached cells were stained with PECAM and smooth muscle actin (SMA) antibodies. In the absence of VEGF, neither population generated PECAM⁺ endothelial cells, but the *Flk1*⁺ cells attached well and developed into a uniform outgrowth of SMA⁺ putative SMCs (Fig. 3A). When VEGF was present, the *Flk1*⁻ cells attached and outgrew into populations of SMA⁻, PECAM⁻ cells (not shown) but the *Flk1*⁺ populations developed mixed cultures of PECAM⁺ and SMA⁺ cells (Fig. 3B).

When plated at a lower density, such that individual colonies could be observed, it was found that *Flk1*⁺ populations gave five classifiable cell types: PECAM⁺ endothelial cells, SMA⁺ SMCs, PECAM/SMA double-positive cells, unattached hematopoietic cells, and unstained attached cells (Fig. 3C). The existence of PECAM/SMA double-positive cells was confirmed by fluorescent immunohistochemistry (Fig. 3D). Because PECAM and SMA are not restricted to endothelial and SMCs, respectively, we confirmed the identities of the cells observed using other markers such as VE-cadherin for endothelial cells and calponin for SMCs (Fig. 3E,F). We also examined whether the PECAM⁺ cells could take up DiI-LDL, which can be incorporated into endothelial cells. PECAM⁺ clusters took up DiI-LDL (Fig. 3E). DiI-LDL⁺ clusters were also VE-cadherin⁺ (Fig. 3F), and SMA⁺ cells were also calponin⁺ (Fig. 3G). These data indicate that it is highly likely that the attached cells in the *Flk1*⁺ cultures are endothelial and SMCs.

We also tested whether the blast colonies derived from single *Flk1*⁺ cells could show the same multipotentiality. Blast colonies, which all produce nonadherent hematopoietic cells, were picked from methylcellulose media after 4 d of culture and plated into individual wells of collagen-coated 96-well plates and cultured in the presence of VEGF. After 1 d, the attached cells were examined by immunohistochemistry with SMA and PECAM antibodies. Some picked blast colonies gave rise to adherent cells that were solely PECAM⁺ (Fig. 4C). The majority showed a mixed pattern of PECAM⁺ and SMA⁺

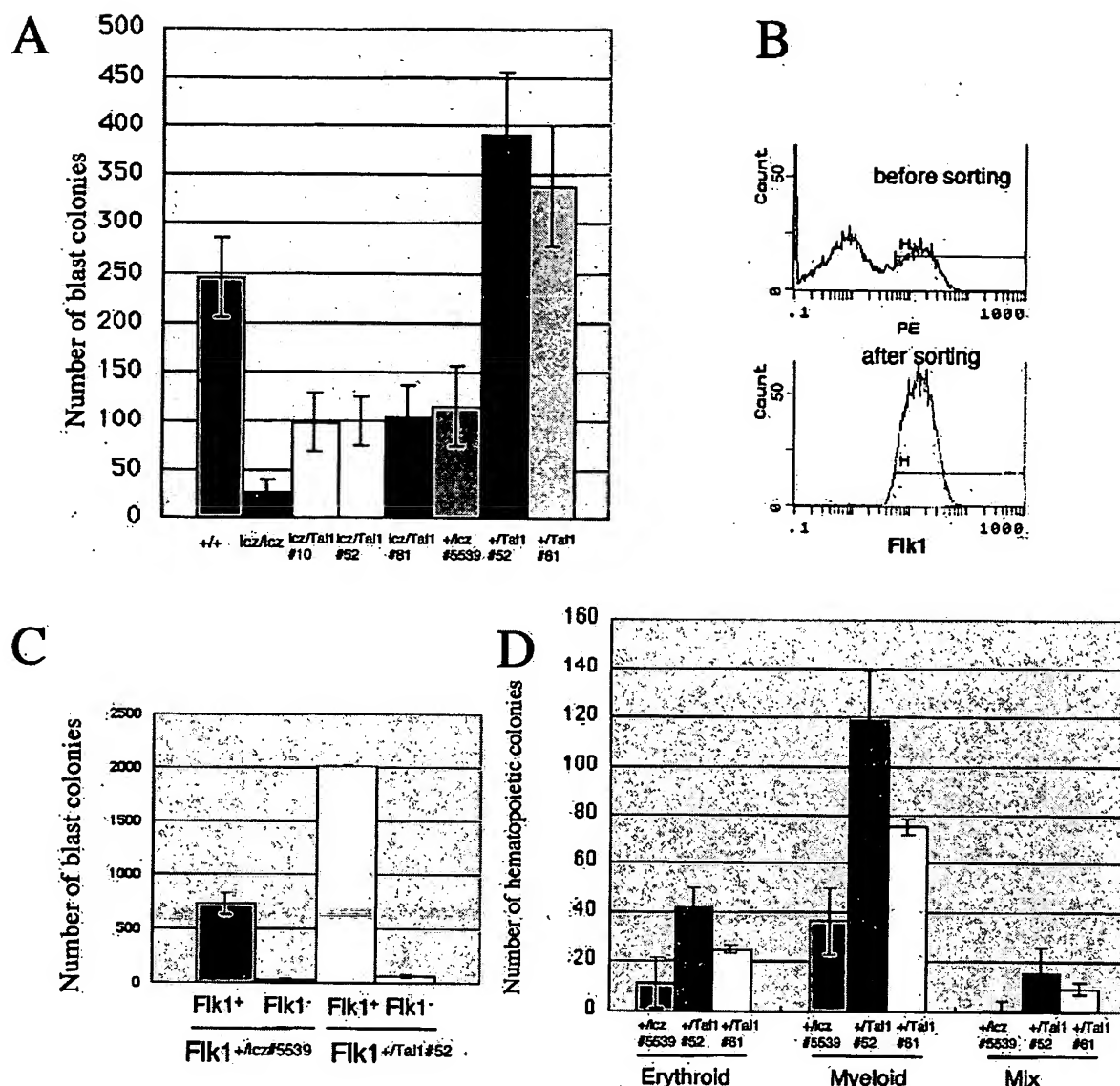


Figure 2. Blast and hematopoietic colony formation in *Tal1*-expressing ES cells. (A) Blast colony assay. Wild-type R1, *Flk1*^{+/lcz}, two independent *Flk1*^{+/Tal1} ES cell lines #52 and #61, *Flk1*^{lcz/lcz} lines, and three independent *Flk1*^{lcz/Tal1} ES cell lines #10, #52, and #81 were subjected to blast colony assay. Blast colonies were counted after 4 d culture. (B) FACS sorting of day 2.75 EB cells with anti-Flk1 antibodies. In the first sort, 35% of cells were Flk1⁺. Resorting of these cells confirmed the purity as 99.2%. (C) Expression of *Tal1* increases the ratio of blast colony-forming units in the Flk1⁺ population. Flk1⁺ and Flk1⁻ cells were sorted from day 2.75 EBs from *Flk1*^{+/lcz} and *Flk1*^{+/Tal1} ES cell lines, and cultured in the presence of VEGF for 4 d. (D) Hematopoietic colony assay. Single-cell suspension from day 7 EBs were cultured in the presence of various cytokines as described in Materials and Methods, and the colonies were counted after 9 d.

cells (Fig. 4A,B), suggesting that blast colonies are heterogeneous but that there can be a common progenitor for hematopoietic, endothelial, and SMCs. Interestingly, after a longer period of culture (1 wk), colonies with only SMCs (Fig. 4F) appeared alongside colonies with only PECAM⁺ cells (Fig. 4E) and colonies with mixed cells (Fig. 4D). The appearance of colonies of pure SMC type from colonies that were all originally at least partially PECAM⁺ could be explained either by selection against

the endothelial component during culture or by transdifferentiation of endothelial cells into SMCs.

Differentiated endothelial cells can make SMA⁺ cells in vitro

To test whether differentiated endothelial cells can still make SMCs under our culture conditions, we sorted Flk1⁺ cells from day 5 wild-type EBs, and double-sorted

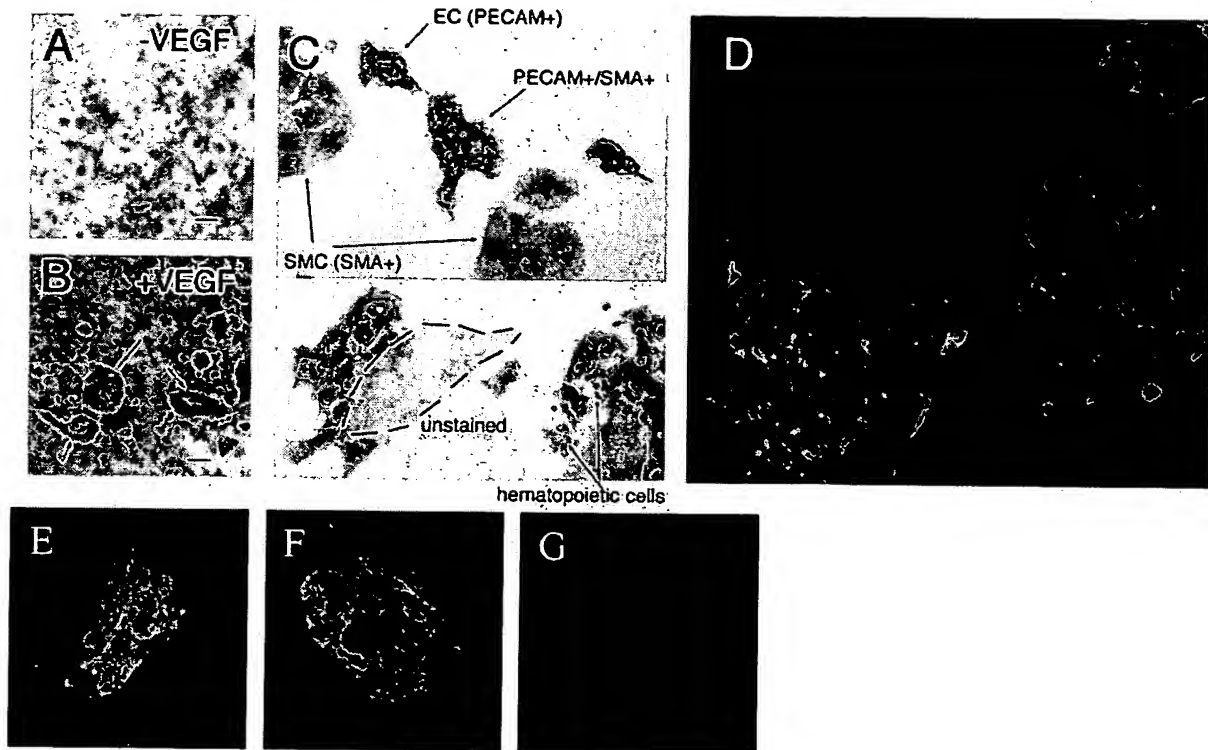


Figure 3. Flk1 marks progenitors for hematopoietic, endothelial, and smooth muscle cells in EB differentiation. The sorted Flk1⁺ cells were cultured in IMDM media in the absence (A) or presence (B) of VEGF (50 ng/mL) on type IV collagen-coated dishes for 3 d and subjected to immunohistochemistry as described in Materials and Methods. (A) In the absence of VEGF, >99% Flk1⁺ cells form SMA⁺ cells (brown). (B) In the presence of VEGF, PECAM⁺ cell clusters (purple) are observed, as are SMA⁺ cells. (C) Five different cell types generated from sorted Flk1⁺ cells: PECAM⁺ endothelial cells (purple, arrows) with cobblestone-like morphology, SMA⁺ SMCs (brown) with flat morphology, PECAM/SMA double-positive cells in brown/purple, hematopoietic cells with rounded morphology, and non-stained cells. (D) Fluorescent immunohistochemistry of the cultured Flk1⁺ cells: PECAM (green), SMA (red), Hoechst nuclear staining (blue). (E) PECAM (green) staining after DiI-LDL (orange) incorporation. (F) VE-cadherin (in green) staining after DiI-LDL (in orange) incorporation. (G) Calponin staining (red) of the cultured Flk1⁺ cells.

them for PECAM expression. By this stage of culture, nearly all Flk1⁺ cells were also positive for the endothelial differentiation markers, PECAM and Tie2 (Fig. 5A). Flk1⁺/PECAM⁺ cells were replated sparsely in 96-well collagen-coated plates at single-cell plating density, and cultured in the presence of VEGF for 3 d (Fig. 5B). We observed that 80% of the single cell-derived colonies were solely SMA⁺ (Fig. 5B), suggesting that cells with the properties of differentiated endothelial cells are still able to differentiate into SMCs. We also sorted Flk1⁺/Tie2⁺ cells from E9.5 mouse embryos. By this stage, such cells are SMA⁺, fully functional endothelial cells. The purity of the double-sorted cells was over 99% (Fig. 5C). When plated at single-cell plating density in 96-well plates in the presence of VEGF, only 15% of colonies remained solely PECAM⁺ after 3 d. Of the remaining colonies, 61% were PECAM⁺/SMA⁺ (Fig. 5D), and 24% were SMA⁺ only (Fig. 5E). Given that the plating efficiency of the starting population is 5% and the purity of the FACS-sorted population is 99%, this strongly suggests that endothelial cells retain the ability to differentiate into SMCs at least during early endothelial differentiation.

TAL1 expression affects endothelial versus SMC differentiation of ES cells

Having established both bulk cultures and single-cell culture conditions to assess the ability of Flk1⁺ cells to generate endothelial and SMCs, we assessed the ability of the Flk1⁺/Tal1 knock-in ES lines to differentiate into these lineages. Flk1⁺ cells were sorted from day 2.75 Flk1⁺/lacZ and Flk1⁺/Tal1 EBs and cultured at low density on collagen-coated plates (Fig. 6A–F). In the absence of VEGF, virtually no endothelial cells were formed by any line. However, in the presence of VEGF, the Flk1⁺ population from Flk1⁺/Tal1 EBs clearly generated more endothelial cells compared to Flk1⁺/lacZ EB cells (Fig. 6A–F). This finding was confirmed by cell counting. There was no overall change in the number of cells in the culture, but rather the ectopic expression of TAL1 increased the number of endothelial cells at the expense of SMCs (Fig. 6G). To assess whether this effect occurred at the level of the single progenitor, blast colony assays were performed. Ectopic TAL1 expression decreased the percentage of colonies with only SMCs and increased the colo-

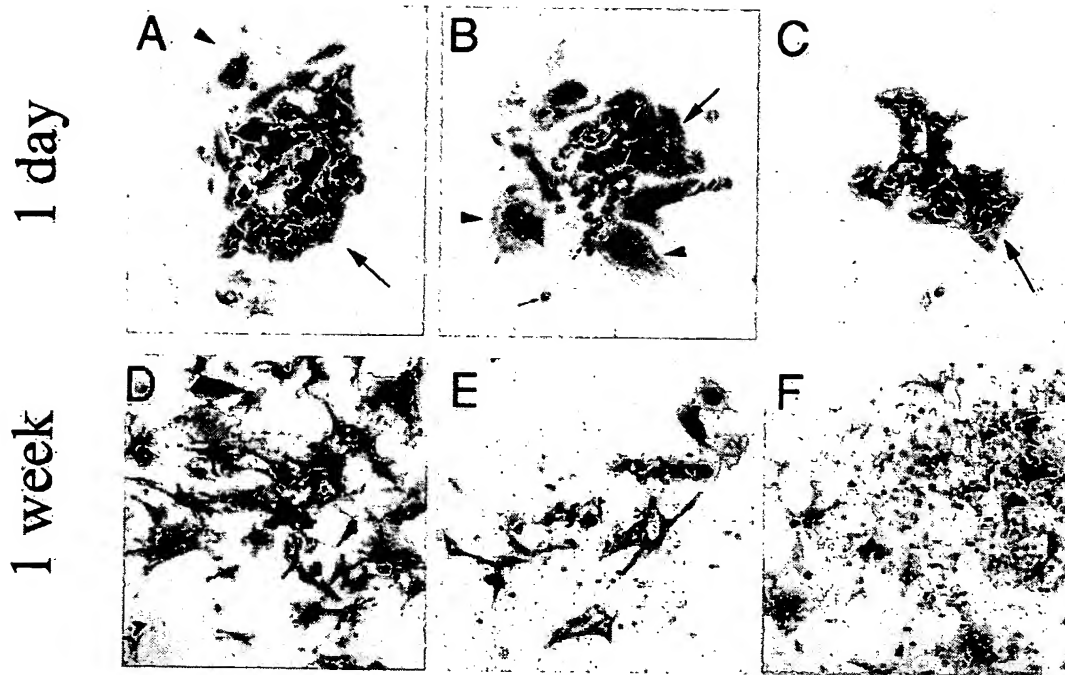


Figure 4. A common progenitor for hematopoietic, endothelial, and smooth muscle cells. Blast colonies were picked from methylcellulose media after 4 d culture into type IV collagen-coated 96-well dishes in the presence of VEGF (50 ng/mL) and cultured further for 1 d (A–C) or 1 wk (D–F). Adherent cells were subjected to immunohistochemistry with PECAM (purple) and SMA (brown), as described in Materials and Methods. Typical colonies containing both endothelial and smooth muscle cells are shown in A and B. Arrowheads indicate SMCs. Arrows indicate PECAM⁺ endothelial cell cluster. Small arrow indicates rounded cells, which are most likely hematopoietic cells. (C) Colony containing only PECAM⁺ endothelial cells. (D) Mixed colony containing PECAM⁺ endothelial cells and SMCs. (E) Colony containing only endothelial cells. (F) Colony containing only SMCs.

nies expressing only PECAM, suggesting that TAL1 can facilitate endothelial differentiation at the expense of smooth muscle (Fig. 6H).

In each assay system, the effect of ectopic TAL1 expression tended to promote endothelial differentiation at the expense of SMC development. However, the effect was never "all-or-none." If the level of TAL1 expression is truly important for endothelial differentiation in these culture conditions, one would predict that ES cells lacking *Tal1* but retaining *Flk1* function would show the reverse effect and be more likely to differentiate into SMA⁺ cells than endothelial cells. This was tested by sorting *Flk1*⁺ cells from day 2.75 EBs derived from *Tal1*^{+/-} and *Tal1*^{-/-} cells and culturing them on collagen plates in the conditions described above. In these conditions, the heterozygous cells generated both SMA⁺ and PECAM⁺ cells, whereas the *Tal1*^{-/-} cells generated only SMA⁺ cells (Fig. 7A–E). The loss of endothelial differentiation in these conditions was fully dependent on the expression of *Tal1*, because null mutant lines in which *Tal1* expression had been rescued by retroviral expression of *Tal1* (Porcher et al. 1996) showed restored ability to generate endothelial cells (Fig. 7D). Single-cell deposition assays were also performed, and over 99% of the colonies generated from *Tal1*^{-/-} ES cells contained smooth muscle only (Fig. 7E). *Tal1*^{-/-} cells are not totally deficient in their ability to generate endothelial cells,

because, as reported earlier (Visvader et al. 1998; Faloony et al. 2000), *Flk1*⁺/PECAM⁺ and *Flk1*⁺/Tie2⁺ cells can be detected when day 10 *Tal1*^{-/-} EBs are FACS-analyzed (Fig. 7F; data not shown).

Discussion

The early development of the endothelial and hematopoietic lineages is closely related in time and space in the vertebrate embryo and may involve a common progenitor, the hemangioblast. Mutation of the VEGF receptor, *Flk1*, leads to loss of both endothelial and hematopoietic lineages from the earliest stage of development in mammalian embryos (Shalaby et al. 1995), consistent with a role in establishing the hemangioblast. The transcription factor TAL1 shows overlapping expression with *Flk1* in the early embryo and has also been proposed to be involved in hemangioblast development (Gering et al. 1998). Mutation of *Tal1* leads to an absence of all embryonic hematopoiesis but early vasculogenesis is unaffected (Robb et al. 1995, 1996; Shivdasani et al. 1995; Porcher et al. 1996), suggesting that *Tal1* plays a critical role in only a subset of derivatives of the early hemangioblasts. However, overexpression of TAL1 in zebrafish embryos caused increased numbers of both hematopoietic and endothelial cells (Gering et al. 1998), and partially rescued the defects of hematopoiesis and vasculo-

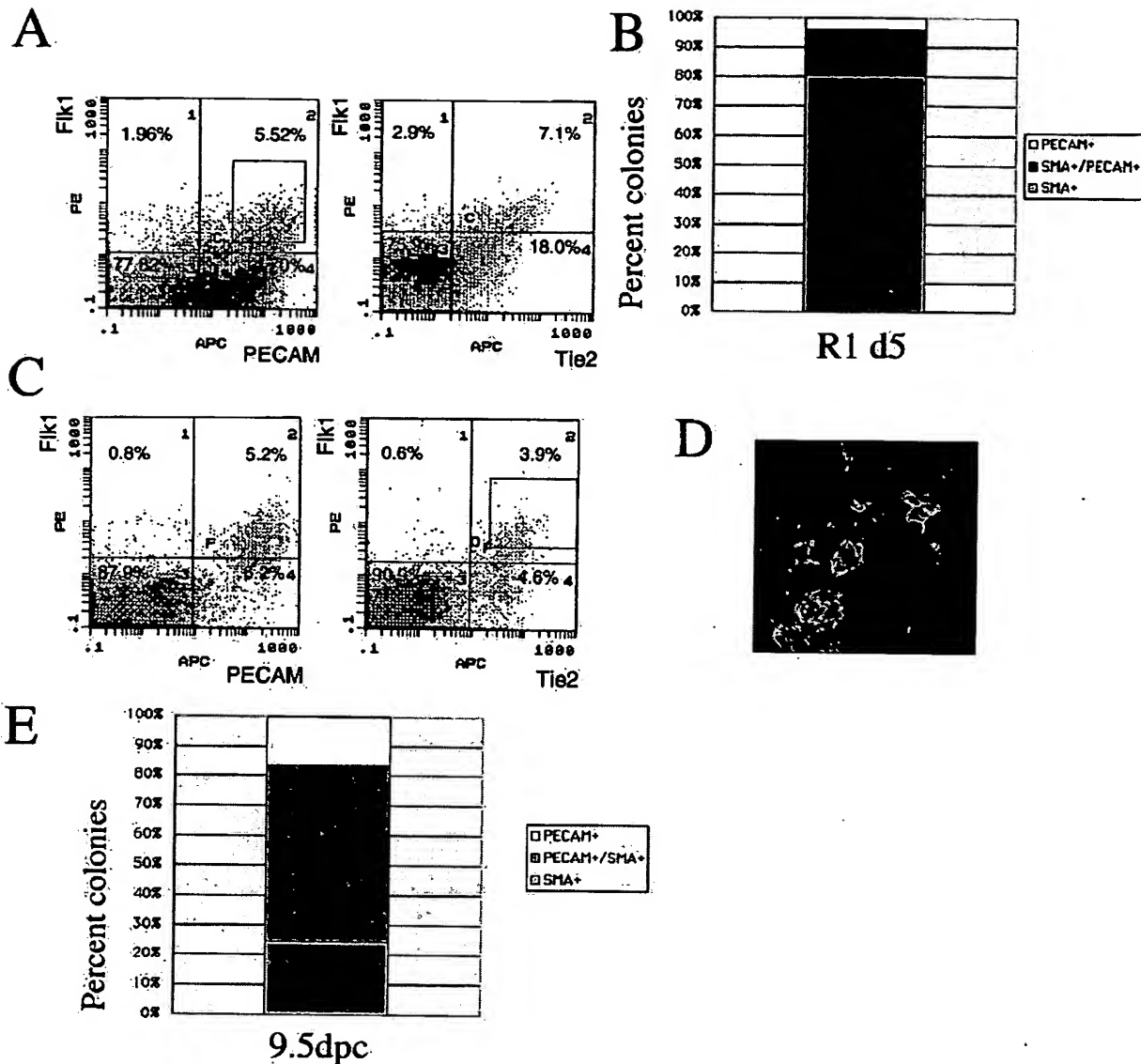


Figure 5. Endothelial cells can transdifferentiate into SMCs. (A) FACS analysis of Flk1/PECAM- or Flk1/Tie2-stained day 5 EB cells. Most Flk1⁺ cells are also positive for PECAM and Tie2. The window used to sort the cells for culture is outlined. (B) Single-cell deposition assay. The Flk1⁺/PECAM⁺ cells from day 5 EBs were sorted and cultured sparsely in 96-well plates for 3 d and subjected to immunohistochemistry for PECAM/SMA expression. Note that 95% of the colonies contain SMA⁺ cells. (C) FACS analysis of Flk1/PECAM- or Flk1/Tie2-stained cells isolated from E9.5 embryos. Almost all Flk1⁺ cells are also positive for PECAM and Tie2. The window used to sort the Flk1⁺/Tie2⁺ cells is outlined. (D) Fluorescent image of the cultured sorted Flk1⁺/Tie2⁺ cells. The sorted cells were cultured for 3 d in the presence of VEGF and stained for PECAM (green) and SMA (red). Nuclei are shown in blue (Hoechst). (E) Single-cell deposition assay. Flk1⁺/PECAM⁺ cells were sorted and cultured sparsely in 96-well plates for 3 d in the presence of VEGF and subjected to immunohistochemistry with PECAM (green) and SMA (red). Note that 84% of the colonies contained SMA⁺ cells.

genesis of the *cloche* mutant (Liao et al. 1998). This led to the proposal that TAL1 acts to specify the hemangioblast lineage. We show here by genetic experiments in mice and mouse ES cells that the level of TAL1 expression does affect the development and differentiation of the hemangioblast precursors but is not sufficient alone to rescue all aspects of the loss of hemangioblast development that occurs in *Flk1* mutants.

When totally ES-derived embryos were made from *Flk1*^{-/-Tal1} ES cells in which the *Tal1* cDNA had been targeted into the *Flk1* locus, embryos still died around E9.5 and failed to develop a functional circulation. No blood vessels were observed in the embryo proper, and only occasional clumps of *Tie1*-expressing cells and hemoglobin-expressing cells were observed in the yolk sac. In vitro blast colony assays on the same ES cells, how-

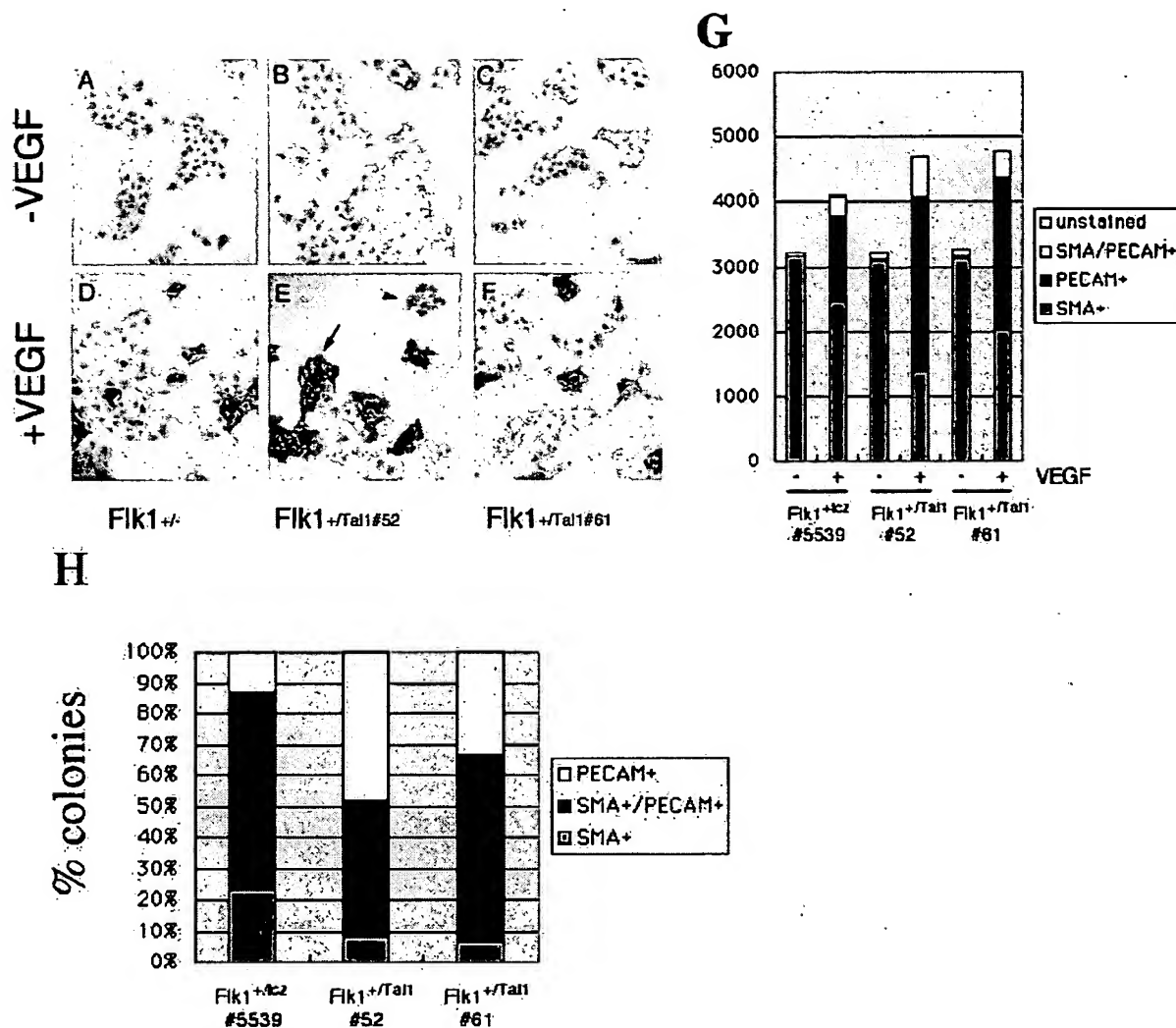


Figure 6. Combinatorial action of VEGF and *Tal1* expression increases number of endothelial cells and decreases number of SMCs. *Flk1⁺* cells from day 2.75 EBs of *Flk1^{+/lacZ}* (A,D), *Flk1^{+/Tal1}* #52 (B,E), and *Flk1^{+/Tal1}* #61 (C,F) EBs were cultured in a 24-well plate in the absence (A–C) or presence (D–F) of VEGF (50 ng/mL) for 3 d, and subjected to immunohistochemistry with PECAM and SMA antibodies. *Tal1*-expressing lines appear to generate more endothelial colonies than the *Flk1^{+/lacZ}* lines. Arrowheads indicate Sma⁺ SMCs. Arrows indicate PECAM⁺ endothelial cells. (G) Quantitation of endothelial/SMC production. Note that *Tal1* knock-in ES cell lines produced a higher number of endothelial cells and fewer SMCs. (H) The distribution of cell-type composition in blast colonies derived from *Tal1*-expressing cells. Blast colonies from *Flk1^{+/lacZ}* and two *Flk1^{+/Tal1}* ES cell lines were cultured for 1 wk in the presence of VEGF and stained with PECAM/SMA antibodies. Both *Flk1^{+/Tal1}* cell lines produced a lower percentage of SMA⁺-containing colonies and more pure endothelial cell colonies.

ever, showed that blast colonies capable of extensive hematopoietic differentiation could develop from the *Tal*-expressing cells in the absence of *Flk1*, in numbers equivalent to *Flk1* heterozygous cells. It was shown previously that, under certain culture conditions, it is possible to obtain some hematopoietic development from *Flk1^{-/-}* ES cells in vitro (Hidaka et al. 1999; Schuh et al. 1999), although this never occurs in vivo. This is thought to reflect a requirement for *Flk1* signaling in promoting correct migration of mesoderm precursors to the yolk sac where primitive hematopoiesis occurs (Shalaby et al. 1997). In vitro culture removes the requirement for mi-

gration and allows some hematopoiesis to proceed. The current experiments show that in vitro hematopoiesis can occur quite normally in *Flk1* mutant ES cells, so long as *TAL1* is expressed in the cells that would normally activate *Flk1*. This suggests that *TAL1* can promote hematopoiesis in the absence of *Flk1* signaling. However, we have not been able to show rescue of vascular development in vivo by the same expression strategy, and we cannot rescue the in vivo lethality. This is probably not a problem in levels of expression, because the *Flk1* promoter seems to be stronger than the *TAL1* promoter (Shalaby et al. 1995; Elefanty et al. 1999). Rather it sug-

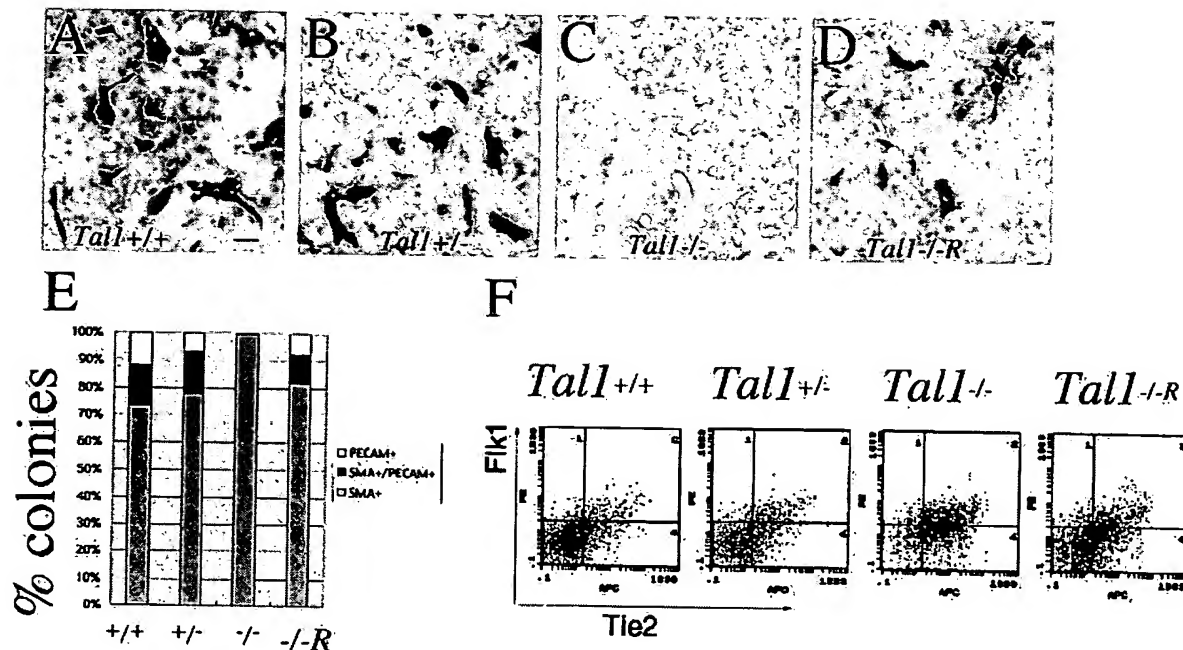


Figure 7. The loss of *Tal1* gene results in reduction of endothelial cell differentiation in collagen cultures. The cultured *Flk1*⁺ cells from day 2.75 wild-type J1 (A), *Tal1*^{+/-} (B), *Tal1*^{-/-} (C), and *Tal1*^{-/-R} (rescued by retroviral expression of *Tal1*; D). EBs were stained for PECAM and SMA. No endothelial cells were seen in the *Tal1*^{-/-} cultures, but were present in the rescued cell lines (E). Quantitation of cell types developing in colonies after single-cell deposition. (F) FACS analysis of day 10 J1, *Tal1*^{+/-}, *Tal1*^{-/-}, and *Tal1*^{-/-R} EBs. *Flk1*⁺/Tie2⁺ cells are formed in *Tal1*^{-/-} EBs.

gests that restoration of *Tal1* expression in *Flk1* mutants is not sufficient to rescue all aspects of *Flk1* signaling required for early development of the vasculature and blood system. In particular, the requirement of *Flk1* for correct migration of vascular and hematopoietic precursors may be independent of the requirement for *Tal1*.

TAL1 has additional effects in the presence of active *Flk1*. When *Flk1*⁺ cells from day 2.75 EBs carrying one active *Flk1* allele and one *Flk1/Tal1* knock-in allele were put into the blast colony assay, a major increase in blast colony formation was observed, compared to both *Flk1*^{-/-} and *Flk1*^{+/-} cells. It was recently shown that only a subset of *Flk1*⁺ cells from early EBs coexpress *TAL1*, and that this population is highly enriched in blast colony progenitors (Chung et al. 2002). Our knock-in strategy ensures that all *Flk1*⁺ cells express *TAL1*, thus essentially increasing the proportion of blast colony-forming progenitors in the population. Because the blast colony has been shown to be a measure of the common progenitor of the endothelial and hematopoietic lineages, both results are consistent with the concept that the hemangioblast is *Flk1*⁺, *TAL1*⁺.

Flk1^{+/Tal1} ES cells also showed altered differentiation in conditions designed to promote endothelial and SMC differentiation. We have confirmed and extended the observations that *Flk1*⁺ cells from EBs can differentiate into cells with properties of vascular smooth muscle, when grown as adherent cells on collagen-based matrices (Yamashita et al. 2000). In the absence of VEGF, essen-

tially all adherent cells became SMCs, but in the presence of VEGF, colonies of PECAM⁺ endothelial cells, SMA⁺ SMCs, and mixed colonies were observed. When the *Flk1*^{+/Tal1} lines were cultured under the same conditions, the differentiation of the *Flk1*-expressing cells was biased towards the endothelial as opposed to the SMC pathway. Conversely, when *Flk1*⁺ cells were sorted from day 2.75 EBs derived from *Tal1*^{-/-} ES cell lines, cultures were almost entirely of the SMC type. Retroviral expression of *TAL1* in these lines rescued endothelial development. This suggests that the level of *Tal1* expression in the *Flk1*⁺ population is important not just for the formation of the hemangioblast but also for its later differentiation. *Flk1*^{+/Tal1} lines have effectively three copies of a potentially active *Tal1* gene, as compared to two copies in wild-type cells, one copy in retrovirus-rescued cells, and no copies in *Tal1*^{-/-} cells. The cell lines show a graded ability to differentiate endothelial versus smooth muscle cells in direct relation to the copy number of the *Tal1* gene.

What is still not clear is the cellular stage at which this effect takes place. In vivo, *Tal1* is not expressed in SMCs and, although it is expressed in early stages of endothelial development (Elefanty et al. 1999; Drake and Fleming 2000), mutants do not show defects in early lineage establishment but do in later vascular remodeling. It is thus not easy to see how *Tal1* expression could act directly to modulate the switch between endothelial and smooth muscle cell fate. Instead, it is possible that the

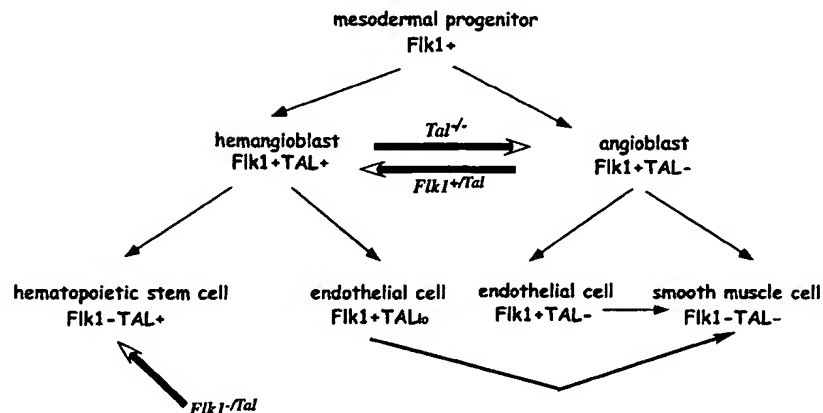
effect is secondary to the role of TAL1 in promoting hemangioblast differentiation from the initial $Flk1^+$ population. Cells that are $Flk1^+, TAL1^+$ can clearly generate both endothelial and hematopoietic cells in vitro, but $Flk1^+, TAL1^-$ cells cannot generate hematopoietic precursors (Chung et al. 2002) and may be restricted to endothelial and smooth muscle development. Nishikawa's group showed that $Flk1^+$ cells from E8.5 mouse embryos could still differentiate into SMA^+ cells in vitro (Yamashita et al. 2000). We show here that highly purified, differentiated, $Flk1^+$ endothelial cells from day 5 EBs or E 9.5 embryos can also differentiate into SMCs in vitro. Similar results were also recently reported for sorted Tie1-positive ES-derived cells (Marchetti et al. 2002). This demonstrates that $Flk1^+$ cells retain the capacity to form SMCs after the time of hematopoietic cell formation. The origin of the SMCs surrounding the developing vasculature in vivo in the intact embryo has been proposed to be largely from neural crest (Le Lievre and Le Douarin 1975) and surrounding mesenchyme (Thayer et al. 1995). There has been some indication from cell marking experiments in chick embryos that vascular endothelial cells can directly contribute to the SMC layers surrounding the developing vasculature by transdifferentiation (DeRuiter et al. 1997). Our in vitro results support that possibility and suggest that there may be both local differentiation and long-range recruitment involved in establishing the supporting lineages of the developing vasculature.

The demonstration that $Flk1^+TAL1^-$ cells can generate both endothelial and smooth muscle cells suggests that there may be two populations of $Flk1^+$ progenitors in ES-derived EBs and the early embryo; the hemangioblast, which is $Flk1^+TAL1^+$ and gives rise to both endothelial and hematopoietic cells, and the angioblast, which is $Flk1^+TAL1^-$ and gives rise to endothelial cells and SMCs. This concept is consistent with studies in the chick embryo which have clearly demonstrated the existence of two types of progenitor by lineage tracing experiments (Pardanaud et al. 1996). We propose a model

(Fig. 8) of progressive specialization of the original $Flk1^+$ cells that develop in EBs or in the early embryo and yolk sac. Initially, $Flk1$ is activated in a population of posterior mesoderm cells at the primitive streak. VEGF signaling promotes the migration of these cells to the yolk sac, where $TAL1$ is activated to generate a population of $Flk1^+TAL1^+$ hemangioblasts. In the embryo proper, $Flk1^+TAL1^-$ angioblast cells develop, which lack hematopoietic potential. As development proceeds, the $Flk1^+TAL1^+$ hemangioblast can down-regulate $Flk1$ to generate $Flk1^-TAL1^+$ hematopoietic cells (Chung et al. 2002). The hemangioblast also generates endothelial cells which are $Flk1^+$ and retain some $TAL1$ expression ($TAL1^{lo}$). The angioblast generates $Flk1^+TAL1^-$ endothelial cells as well as $Flk1^-TAL1^-$ SMCs. Direct development of SMCs from the differentiated $Flk1^+$ endothelial cells, derived from either the hemangioblast or angioblast, may also occur. In this model, the effect of expressing *Tal1* under the *Flk1* promoter is to drive more progenitors into the hemangioblast as opposed to the angioblast pathway. After differentiation, this would then also lead to a greater proportion of endothelial as opposed to smooth muscle cell development. Conversely, the loss of *Tal1* would drive progenitors towards the angioblast pathway and result in the appearance of proportionately more SMCs after differentiation. Finally, expression of *Tal1* under the *Flk1* promoter in *Flk1*^{-/-} cells would short-circuit the requirement for $Flk1$ in the hemangioblast, and allow generation of $Flk1^-TAL1^+$ hematopoietic precursors directly.

This model may be oversimplified in some respects. $TAL1$ expression does not segregate cleanly between hematopoietic and endothelial progenitors and does play some role in vascular development (Visvader et al. 1998). Whether this role is restricted to endothelial cells of hemangioblast origin, as this model would propose, is not known, but is testable. Similarly, it is not yet clear whether SMC differentiation is cleanly segregated from hematopoietic potential as shown in the model. The model applies to the initial separation of endothelial and

Figure 8. A model for the roles of *Flk1* and *Tal1* in regulation of cell fate decision in early hematopoiesis and vascular development. The common $Flk1$ mesodermal progenitor gives rise to two separate lineages: the $Flk1^+TAL1^+$ hemangioblast and the $Flk1^+TAL1^-$ angioblast. These two progenitors both give rise to differentiated endothelial cells, but only the hemangioblast gives rise to hematopoietic cells. The $Flk1^+$ angioblast is proposed to be the direct progenitor of endothelial cells and SMCs. SMCs may also derive by transdifferentiation from the differentiated endothelial cells. The thick arrows indicate the directional effects of the various genetic alterations on lineage development. Gain of function of *Tal1*, in the presence of $Flk1$, biases cells towards the hemangioblast fate, whereas loss of function biases towards the angioblast fate. Ectopic *Tal1* expression in the absence of $Flk1$ bypasses the hemangioblast and leads directly to hematopoietic development.



hematopoietic potential in the early embryo. Whether it also is relevant to the formation of the definitive intra-embryonic hematopoietic stem cells remains to be determined. However, there is a close association of hematopoietic and endothelial progenitors in the aorta-gonad-mesonephros regions and the omphalomesenteric artery (Cormier and Dieterlen-Lievre 1988; Garcia-Porrero et al. 1995; de Bruijn et al. 2000), where definitive hematopoietic progenitors are proposed to arise (Godin et al. 1993; Medvinsky et al. 1993). In addition, both Flk1 (Shalaby et al. 1997) and TAL1 (Porcher et al. 1996; Robb et al. 1996) have been proposed to be required for the onset of definitive hematopoiesis. Proper regulation of both Flk1 signaling and levels of TAL1 expression is clearly important for development of the vasculature, its associated SMCs, and the blood system.

Materials and methods

Construction of the Flk1-Tal1 knock-in vector and establishment of ES cell lines

The *Flk1-Tal1* knock-in vector was constructed as follows. A full-length mouse *Tal1* cDNA (a gift from L. Robb) with a rabbit β -globin polyA signal from the pCAGGS vector was inserted into the first exon of the *Flk1* gene using the previously reported *Flk1* targeting vector (Shalaby et al. 1995). The vector backbone is pPNTloxP, containing PGK-neo flanked by loxP sites and a PGK-tk cassette for negative selection. The *Tal1* cDNA was inserted in the 5' untranslated region of *Flk1* and contains its own initiation codon. The targeting vector was electroporated into R1 ES cells and *Flk1*^{+/lox} ES cells, in which the *neo*^R cassette was removed, to generate *Flk1*^{+/Tal1} and *Flk1*^{lox/Tal1} ES cell lines. The established ES cell lines were verified by Southern blot analysis using the probe shown in Figure 1. Two independent *Flk1*^{+/Tal1} and three *Flk1*^{lox/Tal1} ES cell lines were generated.

ES differentiation, blast colony assay, and hematopoietic colony assay

ES cells were maintained on mouse embryo fibroblast (MEF) feeder cell layers in Dulbecco-modified Eagle medium containing 15% fetal bovine serum, 100 units/mL LIF, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 μ M 2-mercaptoethanol. EBs were generated as described (Kennedy et al. 1997; Choi et al. 1998). In brief, ES cells grown on MEFs were split onto a gelatin-coated dish in Iscove's modified Dulbecco medium (IMDM) with the same supplements as used in the maintenance of ES cells, and were cultured for 1 or 2 d. Single-cell suspensions were then prepared by treatment with 0.25% trypsin and 1.1 mM EDTA, and 6000 ES cells were added per mL to a differentiation medium of IMDM with ascorbic acid (1%), L-glutamine (2 mM), and monothioglycerol (97.5 μ g/mL) on a bacteriological petri dish. For EB formation for myeloid colony assay, conditioned media producing steel factor (KL) and IL-3 were added at final concentration of 1%. After day 2.75, 4.0, or 9.0, a hematopoietic precursor analysis was performed as follows. For the blast colony assay, day 2.75 EBs were trypsinized in prewarmed 0.25% trypsin and 1.1 mM EDTA for 3 min and passed four times through a 20G needle to make a single-cell suspension. Four or five thousand cells per milliliter were replated in methylcellulose (55%) me-

dium containing preselected FBS (10%), ascorbic acid (0.25%), L-glutamine (2 mM), transferrin (1%), MTG, D4T conditioned medium (15%), VEGF (5 ng/mL), and KL (1%). For the primitive erythroid colony assay, erythropoietin (20 U/mL) and protein-free hybridoma medium II (PFHMI, GIBCO-BRL, 5%) were added, and plasma-derived serum (PDS) was added instead of FBS. After 4 d, blast and erythroid colonies were counted. For myeloid colony assays, day 9.0 EBs were treated with 0.25% collagenase for 1 h at 37°C and passed four times through a 20G needle. Six thousand cells per mL were added to methylcellulose (55%) containing L-glutamine (2 mM), transferrin (1%), MTG (97.5 μ g/mL), KL (1%), PFHMI (5%), IL-1 (5 ng/mL), IL-3 (1%), IL-6 (5 ng/mL), IL-11 (5 ng/mL), G-CSF (20 ng/mL), GM-CSF (5 ng/mL), and M-CSF (5 ng/mL), and cultured for 9 d; colonies were then counted.

Hematopoietic colony assays on cells derived from E8.5 embryos were performed as described (Palis et al. 1999). In brief, the yolk sac regions of tetraploid aggregate-derived E8.5 embryos between the late headfold stage and the five-somite stage (before circulation) were dissected out. Tissues were treated with 0.08% collagenase/dispase solution (Boehringer) with 20% FBS for 2 h and transferred into methylcellulose media containing all the prescribed growth factors with the exception of LIF (Palis et al. 1999). The number of colonies was counted after 1 wk.

FACS analysis, sorting, and cell culture

For anti-Flk1 antibody staining, a single-cell suspension was prepared by treating day 2.75 EB cells with trypsin/EDTA solution for 3 min at 37°C. Cells were then treated with mouse serum to block Fc receptors for 15 min on ice. The cells were stained with either phycoerythrin-labeled anti-Flk1 antibody (AVAS12, Pharmingen) or biotinylated anti-Flk1 antibody (a gift from Hans Buhring, University of Tübingen, Germany) for 15 min on ice in the dark, washed once with Hanks-balanced salt solution (HBSS) containing 1% FBS, and stained with secondary antibody (streptavidin-conjugated phycoerythrin, Pharmingen) for 15 min on ice. Cells were washed twice with HBSS containing 1% FBS and suspended in HBSS solution at the concentration of 4×10^6 cells per mL and filtered with 40- μ m nylon mesh before sorting. Cells were sorted on an Epics FACS sorter. Twenty thousand Flk1⁺ cells were sorted and replated onto type IV collagen-coated 24-well dishes and cultured for 3 d in IMDM containing 10% FCS, ascorbic acid, and L-glutamine in the absence or presence of VEGF (50 ng/mL).

Immunohistochemistry

Primary antibodies used were anti-PECAM-1 antibody (MEC13.3, Pharmingen), anti-smooth muscle actin antibody (1A4, Sigma), and anti-VE cadherin (VCAD1, a kind gift from Dr. N. Matsuyoshi, Kyoto University, Japan), anti-calponin (hCP, Sigma). Secondary antibodies were alkaline phosphatase-conjugated anti-rat IgG (Biosource International), horseradish peroxidase-conjugated anti-mouse IgG (ZYMED), Cy3-conjugated anti-rat IgG, and Cy-5-conjugated anti-rat IgG (Jackson Immunological). Immunohistochemistry was carried out using standard procedures. Briefly, cells were fixed in 4% paraformaldehyde at 4°C for 10 min, washed twice with PBS, and treated with H₂O₂ solution (MeOH: 10% Na₂S₂O₈: 30% H₂O₂ = 50:1:10) for 1 h at 4°C. After two 10-min washes with PBS, the cells were treated with blocking reagent (2% skim milk, 1% goat serum, 0.2% bovine serum albumin) for 1 h at 4°C. The cells were incubated with primary antibodies at 1:200 dilution in a blocking solution overnight at 4°C. After washing twice with PBS, 0.05% Tween, 2 mM levamisole, secondary antibodies were

added at 1:200 dilution and incubated for 1 h at room temperature. After washing, the horseradish peroxidase (HRP) color reaction was carried out for 10 min with Diaminobenzidine (Vector Laboratories). After brief washing with NTMT solution (100 mM Tris-HCl at pH 9.5, 0.1 M NaCl, 50 mM MgCl₂, 0.1% Tween), the alkaline phosphatase color reaction was carried out in 4.5 µL/mL of 5-bromo-4 chloro-3-indoxyl phosphate (BCIP) and 3.5 µL/mL of nitro blue tetrazolium chloride (NBT; BCIP/NBT, Boehringer Mannheim) in NTMT for 10–15 min. For the DiI-Ac-LDL uptake experiment, the cultured cells were incubated with 10 µg/mL DiI-Ac-LDL (Molecular Probes) for 4 h and washed in medium, followed by incubation with Hoechst 33342 for 5 min. Fluorescent images were captured on an inverted microscope (Leica DMRXE) and processed by the Openlab system.

Tetraploid aggregation and X-gal staining

Completely ES cell-derived embryos were generated as described (Nagy et al. 1993). The dissected embryos were stained with X-gal as described (Shalaby et al. 1995).

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